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Research Study Application Note

PAXgene® Saliva Collector efficiently stabilizes SARS-CoV-2 derived RNA and dramatically reduces the virus infectivity

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Introduction

During the current COVID-19 pandemic, precise and state-of-the-art testing for the presence of SARS-CoV-2 in research studies is of the utmost importance. While obtaining respiratory samples by nasopharyngeal swabs represented the first method of choice for diagnosis and research, saliva collection for detection of SARS-CoV-2 infection is gaining increasingly more importance. Advantages of saliva samples include non-invasiveness, possibility for convenient at-home collection and unnecessity of medically trained personnel for sample collection (Azzi et al., 2021, Butler-Laporte et al., 2021; Byrne et al., 2020; Pasomsub et al., 2020).

The PAXgene Saliva Collector* is part of a preanalytical workflow comprising human saliva collection, stabilization, transport and storage for nucleic acid extraction and subsequent analyses. The collection device contains 1 ml stabilizing solution which preserves DNA levels in 2 ml human saliva samples by protecting DNA from degradation and inhibiting bacterial growth over storage time. Saliva can be easily collected via a funnel into a plastic tube and mixed with the stabilizing solution upon unscrewing and removal of the funnel.

In the research study presented here, the PAXgene Saliva Collector was tested for its capability to stabilize and reduce infectiousness of active SARS-CoV-2 as part of the Trans National Access project NESARSdia, which was granted by the Advancing European Research Infrastructure on Highly Pathogenic Agents (ERINHA-Advance; Grant Agreement Number: 824061) and funded by the European Commission.

Materials and methods

Samples

Human saliva was obtained by collection in conical tubes from adult, voluntary donors at Medical University Graz, Austria. All donors gave their written informed consent for sample donation. The study was approved by the Ethics Committee of the Medical University Graz under study number 32-666 ex 19/20.

Experimental Setup for Virus Stabilization

In a biosafety level 3 (BSL3) laboratory, viral SARS-CoV-2 copies of Human 2019-nCoV Isolate (Infectious cell culture supernatant of human 2019-nCoV: Human 2019-nCoV ex China Strain: BavPat1/2020 Isolate: Germany ex China; 026V-03883, Institute of Virology, Charité Berlin, Germany) were spiked in human saliva to three different concentrations and mixed by vortexing. A dose control sample was taken directly from spiked-in saliva in order to validate levels of viral RNA. 2 ml saliva samples containing spiked-in virus were directly pipetted into PAXgene Saliva Collectors (PreAnalytiX®, cat. no. 769040) and mixed with the stabilizing solution in the collectors by vortexing resulting in three Collection tubes containing 3 ml of samples with either 1,000, 10,000 or 100,000 viral copies in total. Viral RNA was isolated in triplicates immediately at timepoint 0 and after 24 h and 96 h storage at 20°C in the dark.

Experimental Setup for Virus Infectivity Test

Vero cells at passage 30 were seeded into 48-well plates in concentration of $3x10^4$ cells per well one day prior to infection. On the day of infection, $1.55x10^7$ SARS-CoV-2 copies were spiked in 200 µl PAXgene Saliva stabilizing solution, vortexed and incubated for 10 min at room temperature. As the PAXgene Saliva stabilizing solution is toxic for cells, it was diluted 1:2000 in MEM medium (Gibco, USA, cat. no. 11095-080) without FCS. MEM culture medium was aspirated from adherent cells and 200 µl diluted PAXgene Saliva stabilizing solution containing $6.7x10^4$ SARS-CoV-2 copies was added to the cells for 1 h at 37°C. For control conditions, same number of SARS-CoV-2 was spiked in control medium, diluted and added to the cells. After infection, the PAXgene Saliva stabilizing solution was replaced with standard Vero cell culture medium. Cells were further cultivated for 24 h for immunohistochemical staining and for 72 h for endpoint analyses concerning infection potential of SARS-CoV-2. Besides, morphological analyses of Vero cells after 72 h, supernatant was analyzed by RT-PCR for quantification of SARS-CoV-2 derived RNA.

Isolation and Detection of SARS-CoV-2 derived RNA

Sample aliquots of 140 µl PAXgene Saliva stabilized saliva were processed with QIAamp® viral RNA Mini Kit (QIAGEN®, cat. no. 52904) according to manufacturer's instructions. Obtained SARS-CoV-2 RNA was quantified by RT-PCR using QuantiTect® Probe RT-PCR Kit (QIAGEN, cat. no. 204443), CDC primer N1 (Eurofins, Luxembourg) and Rotor-Gene® Q instrument (QIAGEN, cat. no. 9001580). Calculation of viral copies was performed by including a positive control in every run that was characterized using RNA Standard VR-1986D™ Genomic RNA from 2019 Novel Coronavirus (ATCC, USA).

Immunohistochemistry Staining

Cells were fixed using neutral buffered formalin (10% solution in water with a formaldehyde mass fraction of 3.7%, SAV Liquid Protection, Germany, cat. no. 11723425), permeabilized using Triton-X-100™ (Sigma-Aldrich, USA, cat. no. T9284) and stained with primary antibody Anti-Coronavirus Nucleocapsid Antibody (Sino Biological, China, cat. no. 40143-R019) for 1 h. Secondary antibody Dako REAL™ EnVision™, HRP Rabbit/Mouse (Dako, Denmark, cat. no. K5007) was incubated for 1 h. Secondary antibody was detected using the substrate AEC+ High Sensitivity Substrate Chromogen Ready to use (Dako, Denmark, cat. no. K3469).

Results and discussion

First, stabilization of SARS-CoV-2 derived RNA in the PAXgene Saliva Collector was examined (**Figure 1A**). Obtained results show that viral RNA can be successfully isolated and quantified using QIAamp Viral RNA Mini Kit and QuantiTect Probe RT-PCR Kit on Rotor-Gene Q after saliva storage in PAXgene Saliva Collector. RNA levels remained constant for at least 96 h at 20°C in comparison to dose control, independent of virus numbers that were spiked in (**Figure 1B**).

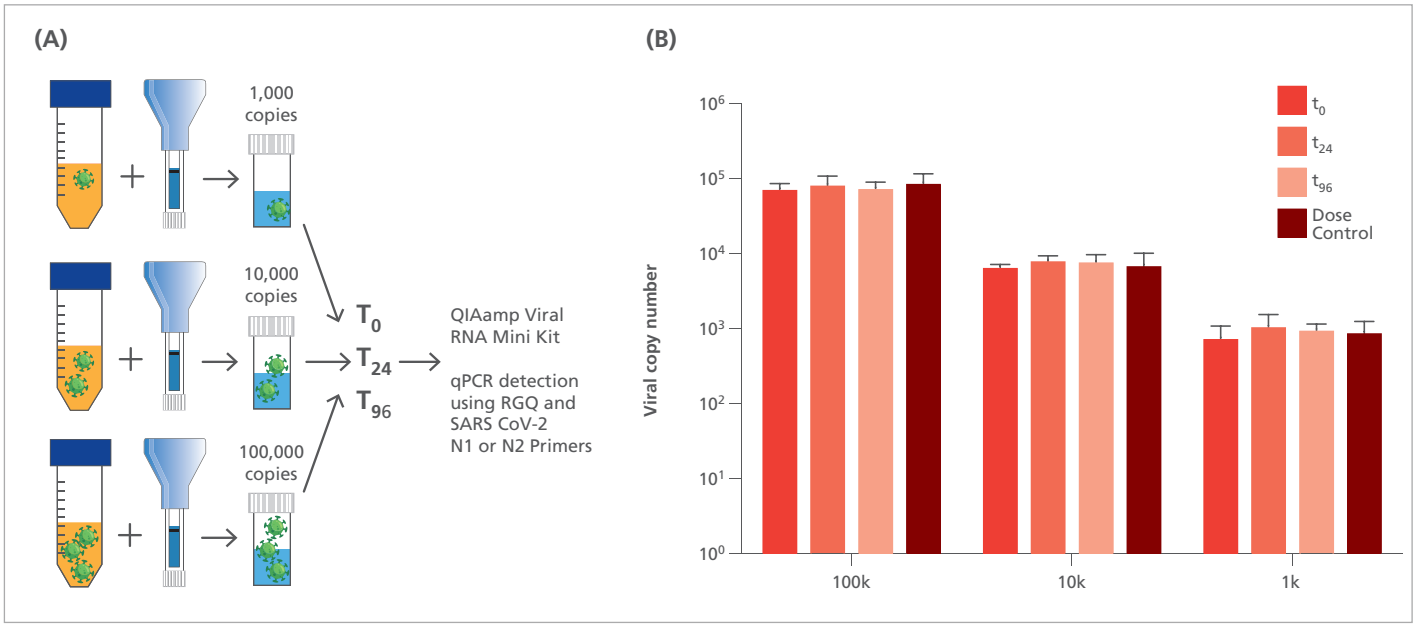

Figure 1: A) Schematic depicting the experimental setup for SARS-CoV-2 derived RNA stabilization. **B)** Quantification of RNA derived from SARS-CoV-2 0, 24 and 96 h after spike-in in PAXgene Saliva stabilizing solution. Experiments were performed in four biological replicates with three technical replicates each; data is represented as mean ± SD.

Next, impact of the PAXgene Saliva stabilizing solution on infectivity of SARS-CoV-2 was examined in a cell culture model (**Figure 2**). Vero cells were treated with diluted stabilizing solution with SARS-CoV-2 spike-in (**Figure 2A Condition 1**), stabilizing solution without SARS-CoV-2 spike-in (**Figure 2A – Condition II**), with medium containing SARS-CoV-2 (**Figure 2A – Condition III**) and with pure medium (**Figure 2A – Condition IV**). An immunohistochemical staining showed that 24 h past infection, SARS-CoV-2 nucleocapsid was not detectable in Vero cells infected with SARS-CoV-2 and treated with PAXgene Saliva Stabilizing Solution. In contrast, the nucleocapsid was abundantly present in Vero cells that were infected with SARS-CoV-2 in medium (**Figure 2B**). Further, 72 h past infection, morphological analyses revealed that Vero cells infected with SARS-CoV-2 and treated

with PAXgene Saliva Stabilizing Solution (Condition I) remained viable, comparable to control conditions II and IV where cells were treated with stabilizing solution or medium only. In contrast, cells incubated with medium containing SARS-CoV-2 (Condition III) were detached and apoptotic, thereby showing clear signs of virus infection (**Figure 2C**). Quantification of viral RNA in supernatant yielded significantly higher C_T values in Condition I where cells infected with SARS-CoV-2 were treated with PAXgene Saliva Stabilizing Solution, in contrast to cells infected with untreated SARS-CoV-2. This was equivalent to a 7 $\log_{10}$ reduction in viral copies in PAXgene Saliva treated samples (**Figure 2D**). For comparison, the efficacy of disinfectants is commonly measured by the fold reduction in infectivity of a pathogen (Lin et al., 2020). The US Food and Drug Administration (FDA) defines a 6 $\log_{10}$ reduction as a high-level disinfectant which corresponds to an extinction of 99.9999% of Mycobacteria (Rutala et al., 2008).

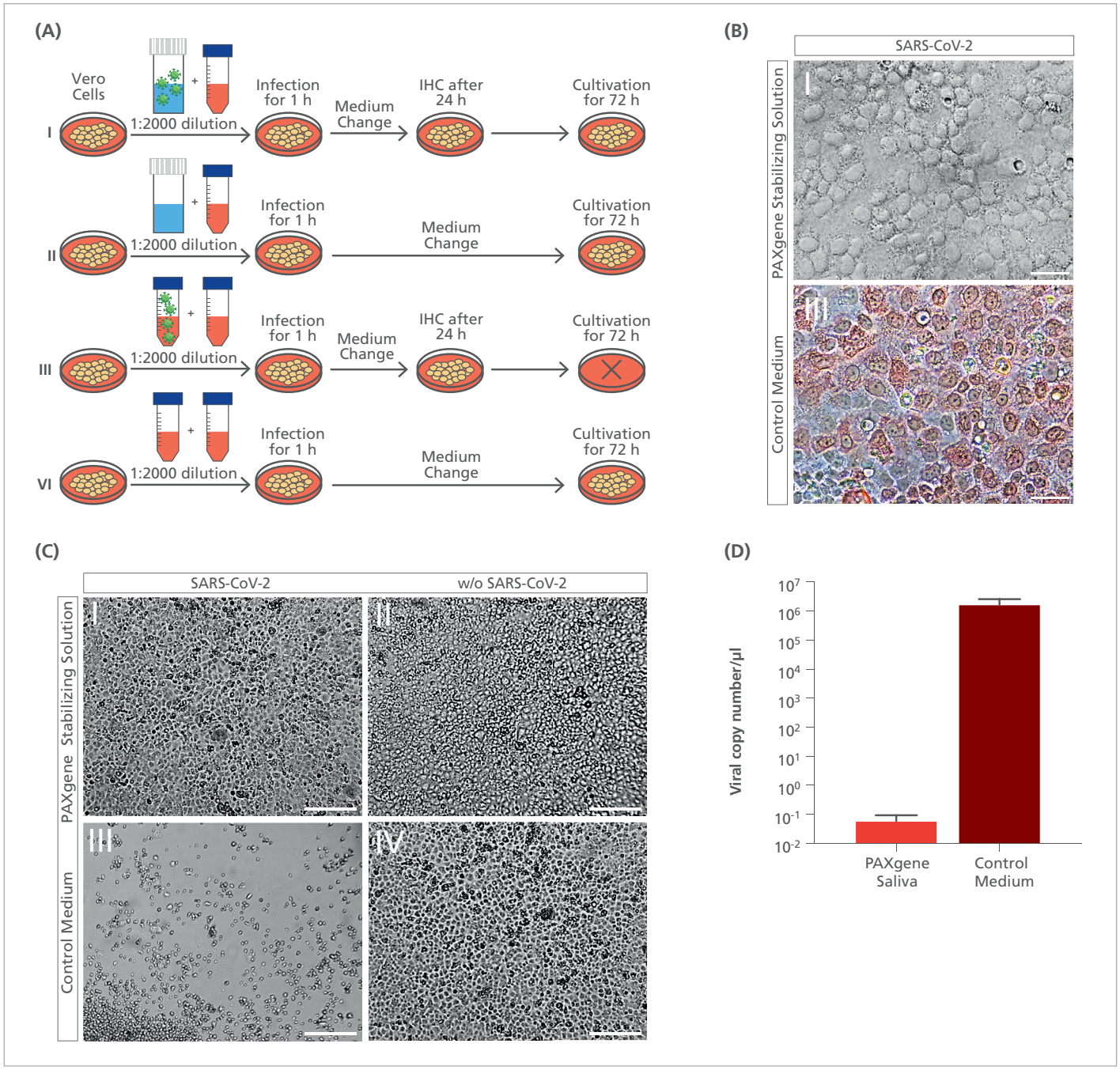

Figure 2: PAXgene Saliva Collector reduces infectivity of SARS-CoV-2
A) Schematic depicting workflow for testing infectiousness of SARS-CoV-2 virus after incubation in PAXgene Saliva Collector for 10 min. **B)** Immunohistochemical staining 24 h after infection of Vero cells with SARS-CoV-2 in PAXgene Saliva stabilizing solution and SARS-CoV-2 in control medium. Scale bar represents 50 µm. **C)** Micrographs showing Vero cells 72 h past infection in four conditions introduced in A. Scale bar represents 250 µm. **D)** Quantification of SARS-CoV-2 derived RNA in the supernatant 72 h after infection using RT-PCR. Experiments were performed in three biological replicates with three technical replicates each; data is represented as mean ± SD.

Summary

Results obtained in this research study could show that SARS-CoV-2 derived RNA is efficiently stabilized for at least 96 h when infectious saliva is collected in the PAXgene Saliva Collector. A time period of up to four days covers the need for sample collection, transport to laboratory and subsequent sample analysis. Moreover, the PAXgene Saliva stabilizing solution prevented infection of Vero cells and reproduction of SARS-CoV-2. Viral RNA copies from potentially infectious virus particles were reduced by 7 $\log_{10}$ in comparison to positive control thereby limiting the risk of infection for laboratory personnel.

In conclusion, the PAXgene Saliva Collector is well-suited for use in SARS-CoV-2 research fields.

References

Azzi L, Maurino V, Baj A, Dani M, d'Aiuto A, Fasano M, Lualdi M, Sessa F, Alberio T. Diagnostic Salivary Tests for SARS-CoV-2. J Dent Res. Feb;100(2):115-123. doi: 10.1177/0022034520969670. Epub 2020 Oct 31. PMID: 33131360; PMCID: PMC7604673, ***2021***

Butler-Laporte G, Lawandi A, Schiller I, et al. Comparison of Saliva and Nasopharyngeal Swab Nucleic Acid Amplification Testing for Detection of SARS-CoV-2: A Systematic Review and Meta-analysis. JAMA Intern Med.;181(3):353–360. doi:10.1001/jamainternmed.2020.8876, ***2021***

Byrne RL, Kay GA, Kontogianni K, Aljayyoussi G, Brown L, Collins AM, et al. Saliva Alternative to Upper Respiratory Swabs for SARS-CoV-2 Diagnosis. Emerg Infect Dis.;26(11):2769-2770. https://doi.org/10.3201/eid2611.203283, ***2020***

Lin Q, Lim JYC, Xue K Yew, PYM, Owh C, Chee PL, Loh XJ; Sanitizing agents for virus inactivation and disinfection. VIEW; 1:e16. https://doi.org/10.1002/viw2.16, ***2020***

Pasomsub E, Watcharananan SP, Boonyawar K, Jacnchompoo P, Wongtabtim G, Suksuwan W, Sungkanuparph S, Phuphuakrat A; Saliva sample as a non-invasive specimen for the diagnosis of coronavirus disease 2019: a cross-sectional study; Clinical Microbiology and Infection, Volume 27, Issue 2, 285.e1 - 285.e4, ***2020***

Rutala, WA, Weber DJ, and the Healthcare Infection Control Practices Advisory Committee (HICPAC)3, Guideline for Disinfection and Sterilization in Healthcare Facilities, ***2008***

Products used

Product		Catalog No.
PAXgene Saliva Collectors (25)*	For 25 individual samples: PAXgene Saliva Collector in a single blister box for 2 ml human saliva collection	769040
Related products		
QIAamp Viral RNA Mini Kit (50)	For 50 RNA preps: 50 QIAamp Mini Spin Columns, carrier RNA, Collection Tubes (2 ml), RNase-free buffers	52904
QIAamp Viral RNA Mini Kit (250)	For 250 RNA preps: 250 QIAamp Mini Spin Columns, Carrier RNA, Collection Tubes (2 ml), RNase-free buffers	52906
QuantiTect Probe RT-PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect Probe RT-PCR Master Mix, 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-Free Water	204443
QuantiTect Probe RT-PCR Kit (1000)	For 1000 x 50 µl reactions: 25 ml 2x QuantiTect Probe RT-PCR Master Mix, 0.5 ml QuantiTect RT Mix, 20 ml RNase-Free Water	204445
Rotor-Gene Q instrument	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1 year warranty on parts and labor, installation and training not included	9001580

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